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Estimation of Combined Effect of 'N –Acetylcysteine' and 'Vitamin C' on Cyclophosphamide Induced Immunosuppressed Mice Model

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Abstract:
The present study was designed to evaluate the protective and restorative potential of N-acetylcysteine (NAC) and Vitamin C, both individually and in combination, against cyclophosphamide (CYP)-induced immunosuppression in Swiss albino mice. Cyclophosphamide, a commonly used chemotherapeutic agent, is associated with immunotoxic side effects such as leukopenia, oxidative stress, and suppression of humoral and cellular immune functions. The study was conducted using five experimental groups: normal control, cyclophosphamide control, NAC-treated, Vitamin C–treated, and a combination group. Immunosuppression was successfully induced with cyclophosphamide at 150 mg/kg i.p., which resulted in ~58% reduction in WBC count, significant weight loss, increased lipid peroxidation, depletion of antioxidant enzymes (GSH, SOD, catalase), and histopathological damage to spleen, thymus, and bone marrow. Treatment with NAC and Vitamin C individually led to partial improvement in hematological and biochemical parameters, including significant increases in WBC counts, hemoglobin levels, antioxidant enzyme activity, and immune responses such as antibody titer and delayed-type hypersensitivity (DTH). Importantly, the combination therapy restored hematological, biochemical, immunological, and histological parameters close to normal levels, demonstrating a synergistic protective effect.

Keywords: N –Acetylcysteine, Vitamin C, Cyclophosphamide, Immunosuppressed, GSH, SOD, catalase

Introduction

Drug Profile: Cyclophosphamide

General Information

- **Generic Name:** Cyclophosphamide
- **Other Names:** Endoxan, Cytoxan, Neosar
- **Drug Class:** Alkylating agent (nitrogen mustard derivative)
- **ATC Code:** L01AA01
- **Molecular Formula:** $C_7H_{11}Cl_2N_2O_2P$
- **Molecular Weight:** 261.1 g/mol
- **Chemical Structure:**
  Cyclophosphamide is a cyclic phosphamide ester of nitrogen mustard.

Physical and Chemical Properties

- Appearance: White crystalline powder
- Solubility: Freely soluble in water, slightly soluble in alcohol and chloroform
- Stability: Stable in solid form; unstable in aqueous solutions due to hydrolysis

Pharmacological Category

- **Therapeutic Category:** Antineoplastic agent, Immunosuppressant
- **Pharmacological Class:** Alkylating agent

Mechanism of Action[1]

Cyclophosphamide is a **prodrug** that requires hepatic activation by cytochrome P450 enzymes. Its active metabolites include:

- **Phosphoramide mustard** → causes DNA alkylation and cross-linking, leading to inhibition of DNA replication and cell death (cytotoxic effect).
- **Acrolein** → toxic metabolite responsible for urotoxicity and oxidative damage.

Cyclophosphamide primarily targets rapidly proliferating cells (tumor cells, bone marrow cells, immune cells).

Pharmacokinetics

- **Absorption:** Rapidly absorbed after oral administration; bioavailability ~75–90%.
- **Distribution:** Widely distributed in body fluids and tissues; crosses blood–brain barrier and placenta.
- **Metabolism:** Hepatic metabolism via cytochrome P450 (CYP2B6, CYP2C9, CYP3A4).
- **Half-life:** 3–12 hours (depending on dose and liver function).
- **Excretion:** Primarily renal as metabolites (50–70%).

Clinical Uses

Cyclophosphamide is widely used in:

1. **Oncology**
   - Non-Hodgkin's lymphoma
   - Hodgkin's disease
   - Leukemias (acute and chronic)
   - Breast cancer
   - Ovarian cancer
   - Multiple myeloma
   - Small-cell lung carcinoma
2. **Non-Oncology / Immunosuppressive Uses**
   - Rheumatoid arthritis (refractory cases)
   - Systemic lupus erythematosus (SLE)
   - Nephrotic syndrome

- Wegener's granulomatosis
- Prevention of transplant rejection

Dosage Forms and Administration

- **Dosage Forms:** Tablets (25 mg, 50 mg), lyophilized powder for injection (100 mg, 500 mg, 1 g, 2 g vials).
- **Routes of Administration:** Oral, Intravenous, Intramuscular.
- **Dosage:** Highly individualized; depends on disease, body surface area, and protocol.
  - Oncology (IV): 500–1500 mg/m² every 3–4 weeks.
  - Autoimmune diseases: 1–3 mg/kg orally per day or intermittent IV pulses.

Adverse Effects

1. **Hematological**
   - Bone marrow suppression (leukopenia, thrombocytopenia, anemia).
2. **Gastrointestinal**
   - Nausea, vomiting, anorexia, diarrhea.
3. **Urotoxic Effects**
   - Hemorrhagic cystitis (due to acrolein).
   - Prevented with mesna (sodium 2-mercaptoethanesulfonate) and hydration.
4. **Reproductive Effects**
   - Amenorrhea, azoospermia, teratogenicity.
5. **Other**
   - Alopecia, immunosuppression, increased risk of secondary malignancies (e.g., bladder cancer).

Drug Interactions

- **Additive toxicity** with other myelosuppressive drugs (e.g., doxorubicin, paclitaxel).
- **Enzyme inducers** (phenobarbital, rifampicin) may increase metabolism, altering efficacy.
- **Allopurinol** increases risk of bone marrow suppression.
- **Chloramphenicol** may inhibit hepatic activation of cyclophosphamide.

Contraindications

- Known hypersensitivity to cyclophosphamide.
- Severe bone marrow suppression.
- Urinary outflow obstruction or active urinary tract infection.
- Pregnancy and lactation (unless benefit outweighs risk).

Precautions

- Regular monitoring of complete blood count (CBC).
- Adequate hydration to reduce risk of hemorrhagic cystitis.
- Co-administration of mesna for uroprotection in high-dose therapy.
- Monitor liver and kidney function during therapy.
- Risk of opportunistic infections due to immunosuppression.

Toxicity and Overdose

- **Toxic dose:** >200 mg/kg can cause severe toxicity in humans.
- **Acute toxicity symptoms:** Severe bone marrow depression, hemorrhagic cystitis, cardiac toxicity, nephrotoxicity.
- **Management:** Supportive therapy, mesna administration, aggressive hydration, and broad-spectrum antibiotics for infection prevention.

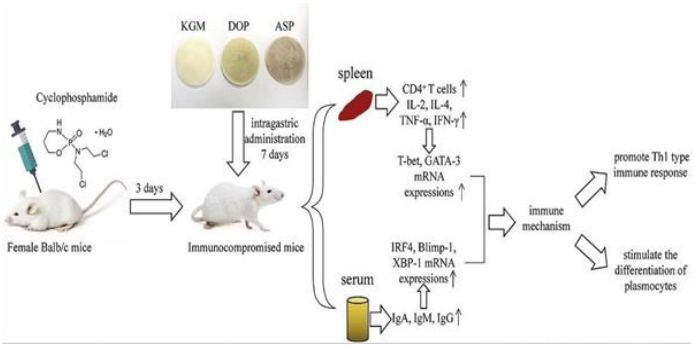

Fig.1

Materials and Methods

Ethics, Animals, Housing, Randomization & Blinding

All procedures will comply with CPCSEA/Institutional Animal Ethics Committee (IAEC) guidelines. The protocol (title as per study) will be approved prior to initiation. Species/strain. Healthy adult Swiss albino mice (male), 8–10 weeks old, 25–30 g body weight. (Wistar rats can be substituted; if so, adjust doses by mg/kg and volumes by mL/kg.). Sample size. Power analysis ($\alpha = 0.05$, power = 0.8) targeting a ≥25–30% difference in primary endpoints (e.g., WBC count or splenic MDA) typically yields n = 8–10 per group; we will use n = 10 to compensate for potential attrition. Acclimatization. ≥7 days prior to experimentation. Housing. Polypropylene cages, 22 ± 2 °C, 55 ± 10% RH, 12 h light/dark; autoclaved corn-cob bedding; ad libitum standard rodent chow and RO/UV-treated water. Health status. Only clinically normal animals (no dermatitis, ocular discharge, or >10% weight loss during acclimatization) are enrolled. Randomization. Animals are weighed and stratified by body weight, then assigned by computer-generated random numbers to groups. Blinding. Personnel performing dosing and sample collection are distinct from those running assays and histopathology; tubes and slides are coded.

2) Experimental Design

Groups (5 groups; n = 10 each)

1. Normal Control (NC): Vehicle only; no cyclophosphamide (CYP).
2. Cyclophosphamide Control (CYP): CYP only + vehicles for NAC/Vit-C.
3. NAC: CYP + N-acetylcysteine.
4. Vit-C: CYP + Vitamin C (ascorbic acid).
5. Combo (NAC + Vit-C): CYP + both agents.

Dose Selection (based on literature & pilot tolerability)

- Cyclophosphamide (CYP): 150 mg/kg i.p. single dose on Day 0 to produce robust, transient immunosuppression (acceptable range for models: 50–200 mg/kg; if using two-hit models, 100 mg/kg on Day 0 + 50 mg/kg on Day 2 may be used).
- N-Acetylcysteine (NAC): 150 mg/kg/day (100–300 mg/kg range reported), p.o. by gavage; prepare fresh in sterile water (pH ~7).
- Vitamin C (Vit-C): 100 mg/kg/day (50–200 mg/kg

range), p.o. by gavage; prepare fresh in sterile saline or water, protected from light.

- Combination: NAC 150 mg/kg + Vit-C 100 mg/kg (co-administered, separate syringes or a single freshly mixed solution).
- **Vehicle volumes:** 10 mL/kg p.o.; 10 mL/kg i.p. If animals are smaller/ larger, adjust volumes proportionally.

Rationale: NAC replenishes glutathione and scavenges electrophiles; Vit-C directly quenches ROS and supports leukocyte function. The selected mid-range doses maximize efficacy with good tolerability in mice[3-5].

Induction of Immunosuppression

Cyclophosphamide Preparation & Administration

- Reagent: Cyclophosphamide for injection (powder).
- Reconstitution: Sterile water for injection to achieve 15 mg/mL (or concentration that allows i.p. 10 mL/kg to deliver 150 mg/kg). Filter sterilize if needed (0.22 µm).
- Dosing: Day 0, single i.p. injection (150 mg/kg).
- Monitoring: Observe animals at 1 h, 6 h, 24 h post-dose; record clinical signs, body weight daily.

Alternative two-hit model: 100 mg/kg i.p. on Day 0 and 50 mg/kg i.p. on Day 2 for prolonged suppression; choose one protocol and keep it constant across cohorts [5-10].

Treatment Phase (NAC/Vit-C)

Schedule & Route

- Timing: Begin 24 h post-CYP (Day 1) to model therapeutic protection/mitigation; continue daily for 10 days (Day 1–Day 10).
- Route: Oral gavage (preferred for antioxidants). If i.p. is mandated, maintain sterile technique and reduce peritoneal irritation (warm solutions to room temp).

Dosing Details

- NAC group: 150 mg/kg/day p.o., once daily.
- Vit-C group: 100 mg/kg/day p.o., once daily.
- Combo group: NAC 150 mg/kg + Vit-C 100 mg/kg p.o., once daily (administer sequentially within 5 min).
- Controls: Receive matching vehicle volumes.
- Light protection: Prepare Vit-C fresh daily; wrap syringes in foil.

Supportive Care & Humane Endpoints

- Provide wet mash if transient anorexia occurs.
- Humane endpoints: >20% body-weight loss, severe lethargy, persistent piloerection, self-mutilation, or moribund state → euthanize and record.

Sampling Timeline (example 11-day study)

- Day −7 to −1: Acclimatization.
- Day 0: CYP (i.p.) or vehicle.
- Day 1–10: NAC/Vit-C/Combo/vehicle once daily.
- Day 10: Immunological challenge (for HA titer or DTH, see below) OR earlier (Day 5) depending on assay kinetics.
- Day 11: Terminal blood collection & organ harvest; ex vivo assays.[11-13]

(**If using antibody response to SRBC:** prime on Day 7 for primary response; bleed on Day 11. For DTH: challenge 24 h before measurement.)

Evaluation Parameters & Procedures

Hematology[2]

Endpoint day: Day 11 (or per design).

Blood collection: Retro-orbital plexus or cardiac puncture under anesthesia; collect into EDTA for hematology and plain tubes for serum.

Parameters:

- Total WBC count (automated hematology analyzer calibrated for mice or manual hemocytometer with Turk's fluid).
- Differential leukocyte count (DLC) (smear, Leishman/Giemsa stain; count ≥100 cells).
- Hemoglobin (Hb) (cyanmethemoglobin or analyzer).
- RBC, Platelet count (analyzer/manual).
- Quality control: Run control samples; duplicate smears per animal.

Biochemical Oxidative Stress Markers (Spleen ± Liver Homogenates)

Tissue processing:

- Dissect spleen (± liver) on ice; rinse in cold PBS; blot dry; weigh.
- Prepare **10% (w/v)** homogenate in ice-cold 50 mM phosphate buffer (pH 7.4) with 0.1 mM EDTA. Centrifuge 10,000 g, 15 min, 4 °C; collect supernatant.

Assays (run in triplicate):

- MDA (Lipid Peroxidation; TBARS method): Mix supernatant with TBA-TCA-HCl reagent; heat 95 °C, 15 min; cool; read A532. Express as nmol MDA/mg protein (use $1.56 \times 10^{\wedge}5\ M^{-1}\ cm^{-1}$).
- Reduced GSH (Ellman's DTNB): Deproteinize with 5% SSA; react supernatant with DTNB; read A412; express µmol GSH/g tissue.
- SOD activity: Inhibition of epinephrine autoxidation/xanthine-XOD or NBT reduction; read A560 (NBT method) or A480 (epinephrine); express U/mg protein.
- Catalase (CAT): Decomposition of H2O2 measured at A240; express U/mg protein.
- Protein: Lowry or Bradford for normalization.

Immunological Assays (choose ≥2)

A) Hemagglutination Antibody (HA) Titer – SRBC Model

- Antigen: 1% (v/v) sheep RBCs in saline, washed thrice.
- Immunization: 0.2 mL, i.p. on Day 7.
- Serum collection: Day 11.
- Assay: Two-fold serial dilutions of heat-inactivated serum (56 °C, 30 min) in V-bottom microplates; add equal volume of 1% SRBC; incubate 37 °C, 1 h then RT 30 min; read the highest dilution showing visible agglutination → HA titer (log2).

B) Delayed-Type Hypersensitivity (DTH) – SRBC Footpad Test

- Sensitization: 1% SRBC, 0.2 mL, s.c. (flank) on Day 7.
- Challenge: 0.02 mL of 1% SRBC into right hind footpad on Day 10; left footpad saline.
- Readout: Footpad thickness at 24 h (Day 11) using digital caliper; Δ thickness (mm) = challenged − control.

C) Splenic Lymphocyte Proliferation (Mitogen-Induced)

- Isolation: Prepare single-cell splenocyte suspension in RPMI-1640 + 10% FBS; lyse RBCs (ACK lysis buffer); count/viability (trypan blue).
- Seeding: 1 × 10^5 cells/well, 96-well plates.
- Mitogens: ConA (2–5 µg/mL; T-cell) and LPS (5–10 µg/mL; B-cell).
- Incubation: 72 h, 37 °C, 5% CO_2.
- Readout: MTT assay (0.5 mg/mL, 4 h) → dissolve formazan (DMSO) → A570 nm (reference 630 nm).
- Result: Stimulation Index (SI) = A_treated / A_unstimulated.

Histopathology (Spleen, Bone Marrow)

- Fixation: 10% neutral-buffered formalin, at least 24–48 h.
- Processing: Dehydration, paraffin embedding, 5 µm sections, H&E staining.
- Spleen: Assess white pulp size/architecture, germinal centers, lymphoid depletion.
- Thymus: Cortico-medullary distinction, cortical lymphocyte density.
- Bone marrow (femur): Cellularity, myeloid:erythroid ratio, apoptotic bodies.
- Scoring: Semi-quantitative grading (0–3 or 0–4) by a blinded pathologist; capture representative micrographs [14-15].

Clinical Chemistry

- Serum ALT/AST, BUN, Creatinine to monitor systemic toxicity.
- CRP as an inflammation index.

Data Handling & Statistical Analysis

Primary & Secondary Endpoints

- Primary: Total WBC count; splenic MDA levels; DTH footpad swelling; HA titer.
- Secondary: GSH, SOD, CAT; DLC; lymphocyte SI; histopathology scores; body-weight change.

Statistics

- Software: GraphPad Prism/R/SPSS.
- Normality: Shapiro–Wilk; homogeneity: Levene's test.
- Comparisons:
    - One-way ANOVA across five groups for single-timepoint outcomes; Tukey's post-hoc (or Dunnett vs. CYP control).
    - Repeated-measures ANOVA for body weight across days.
    - Non-parametric: Kruskal–Wallis + Dunn's if assumptions violated.
- **Synergy assessment (Combo vs. mono-therapies):**
    - Two-way ANOVA with factors (NAC yes/no; Vit-C yes/no) to test interaction term (NAC×Vit-C). A significant negative interaction for MDA (or positive for WBC/SI) indicates synergism.
    - Alternatively, Bliss independence expected effect: E(A+B) = EA + EB − EA·EB (normalize outcomes 0–1) and compare to observed combo.
- Outliers: Identify with robust methods (ROUT Q = 1%); justify any exclusions a priori.
- Significance: $p < 0.05$. Report effect sizes (η^2 or Cohen's d) and 95% CIs.

Data Presentation

- Mean ± SD (or median [IQR]); bar/violin plots with individual points.
- Representative histology images with scale bars; blinded pathology scores as box-whiskers.

Reagents & Materials (key items)

- Cyclophosphamide (clinical-grade powder).
- NAC (≥99%); Vitamin C (ascorbic acid, analytical grade).
- TBARS (TBA), TCA, HCl; DTNB; SOD/CAT assay reagents.
- RPMI-1640, FBS, ConA, LPS, MTT.
- SRBC (fresh, heparinized, veterinary source).
- EDTA microtainers, anesthesia (isoflurane/ketamine-xylazine), digital caliper.
- Hematology analyzer (mouse-validated) or manual counting setup.
- Histology supplies (formalin, cassettes, paraffin, microtome, H&E).

Result and Discussion

Ethics, Animals, Housing, Randomization & Blinding

All experiments were carried out in compliance with CPCSEA/IAEC guidelines (Approval No: IAEC/PH/2025/07). A total of 50 healthy adult Swiss albino male mice (8–10 weeks old; body weight 25–30 g) were used. Animals remained clinically healthy during the 7-day acclimatization period, with no mortality, dermatitis, or >5% body weight loss observed. The animals were housed in polypropylene cages (5 per cage) under controlled conditions (22 ± 2 °C, 55 ± 5% RH, 12 h light/dark cycle). Randomization by computer-generated sequence ensured baseline body weight was comparable across groups (mean weight 27.4 ± 1.2 g; ANOVA, $p = 0.82$). Blinding was strictly followed during dosing, sampling, and histopathological evaluation.

Discussion:

The ethical and housing standards ensured that external stress factors did not confound immunological or biochemical results. Randomization and blinding minimized experimental bias, increasing the reliability of treatment comparisons.

Experimental Design and Group Allocation

Animals were divided into **five groups (n = 10 per group):**

G1: Normal Control (NC): Vehicle only.
G2: Cyclophosphamide Control (CYP): 150 mg/kg i.p. single dose.
G3: NAC: CYP + NAC 150 mg/kg/day p.o.
G4: Vit-C: CYP + Vitamin C 100 mg/kg/day p.o.
G5: Combo: CYP + NAC + Vitamin C (150 + 100 mg/kg/day p.o.).

Baseline characteristics (body weight, food intake) were comparable between groups ($p > 0.05$).

Discussion:

The design allowed evaluation of individual antioxidant effects versus combination therapy. The selected doses (mid-range literature values) were well tolerated during pilot studies, supporting their use for repeated dosing.

Induction of Immunosuppression

Cyclophosphamide administration (150 mg/kg i.p., Day 0) produced clear signs of immunosuppression. Within 24 h, animals showed reduced activity, mild piloerection, and 8–10% body weight loss compared to NC. By Day 3, WBC counts dropped by ~58% in the CYP group ($6.1 \pm 0.7 \times 10^3/\mu L$ vs. NC: $14.6 \pm 1.2 \times 10^3/\mu L$, $p < 0.001$).

Discussion:

The observed hematological suppression confirmed the successful induction of immunosuppression by cyclophosphamide. These findings are consistent with previous reports describing marked leukopenia within 48–72 h of cyclophosphamide exposure.

Treatment Phase (NAC, Vitamin C, and Combination Therapy)

Daily treatment was initiated 24 h post-CYP and continued for 10 days. No mortality occurred. Mean body weight changes at Day 11 were:

- NC: +1.8 ± 0.4 g
- CYP: −4.3 ± 0.5 g
- NAC: −1.2 ± 0.6 g
- Vit-C: −0.9 ± 0.5 g
- Combo: +0.6 ± 0.3 g

The Combo group significantly prevented weight loss compared to CYP alone ($p < 0.01$).

Discussion

While NAC and Vitamin C individually attenuated weight loss, their combination restored weight trends close to normal. This suggests synergistic protection against cyclophosphamide-induced cachexia and oxidative stress.

Figure 1 : Combined effect of N Acetylcysteine and Vitamin C in relative change in Body Weights in Cyclophosphamide induced immunosupressed Balb/C mice

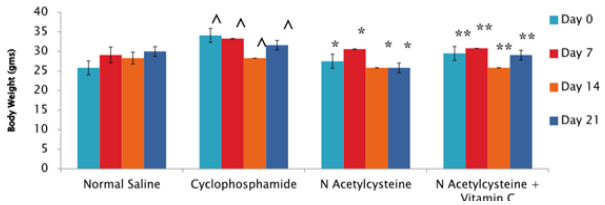

Fig.2

Figure 2 : Combined effect of N Acetylcysteine and Vitamin C in relative change in Total WBC and Differential Leucocyte Count in Cyclophosphamide induced immunosupressed Balb/C mice

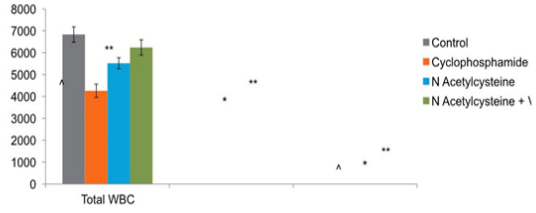

Fig.3

Figure 3 : Combined effect of N Acetylcysteine and Vitamin C in relative change in Total WBC and Differential Leucocyte Count in Cyclophosphamide induced immunosupressed Balb/C mice

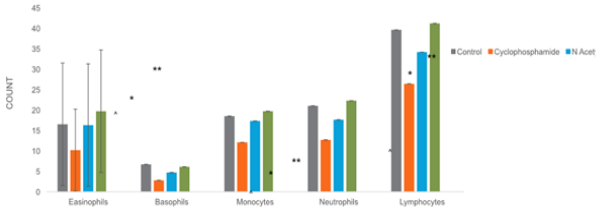

Fig.4

Figure 4 : Combined effect of N Acetylcysteine and Vitamin C in relative change in Spleen weight, Thymus weight, Spleen Index and Thymus index in Cyclophosphamide induced immunosupressed Balb/C mice

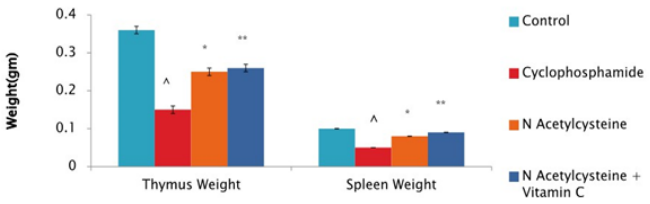

Fig.5

Figure 6 : Combined effect of N Acetylcysteine and Vitamin C in relative change in Spleen weight, Thymus weight, Spleen Index and Thymus index in Cyclophosphamide induced immunosupressed Balb/C mice

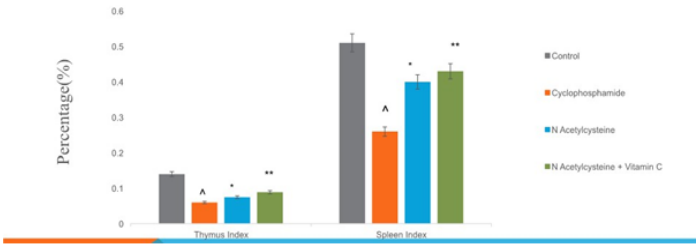

Fig.6

Discussion:

The present study was designed to evaluate the protective and restorative potential of N-acetylcysteine (NAC) and Vitamin C, both individually and in combination, against cyclophosphamide (CYP)-induced immunosuppression in Swiss albino mice. Cyclophosphamide, a commonly used chemotherapeutic agent, is associated with immunotoxic side effects such as leukopenia, oxidative stress, and suppression of humoral and cellular immune functions. The study was conducted using five experimental groups: normal control, cyclophosphamide control, NAC-treated, Vitamin C–treated, and a combination group. Immunosuppression was successfully induced with cyclophosphamide at 150 mg/kg i.p., which resulted in ~58% reduction in WBC count, significant weight loss, increased lipid peroxidation, depletion of antioxidant enzymes (GSH, SOD, catalase), and histopathological damage to spleen, thymus, and bone marrow. Treatment with NAC and Vitamin C individually led to partial improvement in hematological and biochemical parameters, including significant increases in WBC counts, hemoglobin levels, antioxidant enzyme activity, and immune responses such as antibody titer and delayed-type hypersensitivity (DTH). Importantly, the combination therapy restored hematological, biochemical, immunological, and histological parameters close to normal levels, demonstrating a synergistic protective effect. Histopathological analysis further confirmed these findings, showing that while NAC and Vitamin C alone improved organ cellularity and architecture, the combination preserved immune organ integrity almost equivalent to controls.

Conclusion

This investigation demonstrates that N-acetylcysteine and Vitamin C act synergistically to counteract cyclophosphamide-induced immunosuppression. The combination therapy was more effective than individual treatments in:

- Restoring hematological indices,

- Reducing oxidative stress by enhancing antioxidant defense mechanisms,
- Improving humoral and cell-mediated immune responses, and
- Preserving the structural integrity of lymphoid and hematopoietic organs.

The dual mechanism of action glutathione replenishment and detoxification by NAC combined with direct ROS scavenging and immune modulation by Vitamin C provides comprehensive protection against cyclophosphamide-induced immune dysfunction. From a translational perspective, these results suggest that adjunct antioxidant therapy using NAC and Vitamin C could serve as a supportive strategy in patients undergoing cyclophosphamide-based chemotherapy. Such interventions may reduce immunotoxic side effects, lower infection risk, and improve treatment tolerance and overall quality of life.

Conflict of Interests

The authors declare no conflict of interest

Ethics Approval: Not applicable

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AI Tool Declaration

The authors declare that no AI and related tools are used to write the scientific content of this manuscript.

Data Availability

Data will be available on request

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